

# EXHIBIT C

# Endothelial Cell Dysfunction Leading to Diabetic Nephropathy Focus on Nitric Oxide

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**Abstract**—Clinical manifestations of diabetic nephropathy are an expression of diabetic microangiopathy. This review revisits the previously proposed Steno hypothesis and advances our hypothesis that development of endothelial cell dysfunction represents a common pathophysiological pathway of diabetic complications. Specifically, the ability of glucose to scavenge nitric oxide is proposed as the initiation phase of endothelial dysfunction. Gradual accumulation of advanced glycated end products and induction of plasminogen activator inhibitor-1, resulting in the decreased expression of endothelial nitric oxide synthase and reduced generation of nitric oxide, are proposed to be pathophysiologically critical for the maintenance phase of endothelial dysfunction. The proposed conceptual shift toward the role of endothelial dysfunction in diabetic complications may provide new strategies for their prevention. (*Hypertension*. 2001;37[part 2]:744-748.)

**Key Words:** nitric oxide synthase ■ collagen ■ plasminogen ■ diabetes mellitus

Type 2 diabetes mellitus has reached epidemic proportions, and one of its ominous complications, diabetic nephropathy, represents today the leading cause of end-stage renal failure.<sup>1,2</sup> Clinical manifestations of diabetic nephropathy include microalbuminuria, heralding incipient nephropathy, followed by albuminuria or nephrotic-range proteinuria, elevated blood pressure, development of glomerulosclerosis, tubulointerstitial fibrosis, and relentless decline in glomerular filtration rate.<sup>1-4</sup> The most characteristic prognostic feature in this group of patients is the high risk of cardiovascular complications, much more so than in diabetics without nephropathy. Several important mediators of diabetic nephropathy have been proposed, such as transforming growth factor- $\beta$ , accumulation of the extracellular matrix, reactive oxygen intermediates, and protein kinase C, to name a few, and have been comprehensively reviewed.<sup>1-4</sup> However, the pathophysiological origin of clinical presentations of diabetic nephropathy can be traced back to microvasculopathy and macrovasculopathy (Figure 1), and the focus of this review will be on the mechanisms for development and maintenance of endothelial cell dysfunction in diabetic nephropathy.

In 1988, Torsten Deckert delivered a Claude Bernard Lecture in which the Steno hypothesis, a unifying proposal that albuminuria of diabetic nephropathy is a sign of the global vascular dysfunction, was introduced.<sup>5</sup> Because only less than one third of patients with type I diabetes mellitus tend to develop renal disease, it was speculated that a genetic predisposition, supposedly at the level of *N*-deacetylase (a key enzyme responsible for the sulfation of heparan sulfate

proteoglycans) gene polymorphism, contributes to the loss of the anionic charge barrier of endothelial cells and basement membranes, resulting in a widespread rise in vascular permeability and vasculopathy. The hypothesis to be developed below takes stock of the above broad view that albuminuria is an indicator of a systemic microvascular lesion in diabetes mellitus and revises the Steno hypothesis to advance a hypothesis on the developmental mechanisms of endothelial cell dysfunction in diabetes.

### The Concept of Endothelial Cell Dysfunction

The last 20 years have brought about a lucid realization that the vascular endothelium is not a mere barrier between intravascular and interstitial compartments. In fact, the vascular endothelium has received the status of an organ, albeit a widely spread one, which is responsible for the regulation of the hemodynamics, angiogenic vascular remodeling, and metabolic, synthetic, inflammatory, antithrombotic, and prothrombotic processes. As any other organ, the vascular endothelium is a subject for dysregulation, dysfunction, insufficiency, and failure. This latter category has become the basis for the recently coined syndrome of endothelial cell dysfunction (ECD). This syndrome, initially introduced to describe defective endothelium-dependent vasorelaxation in patients at risk for development of atherosclerosis even before angiographic or ultrasonographic evidence of the disease becomes detectable<sup>6-8</sup> (reviewed in Reference 9 and 10),<sup>9,10</sup> has been broadened to encompass disturbances in the barrier function of the vascular endothelium; its impaired

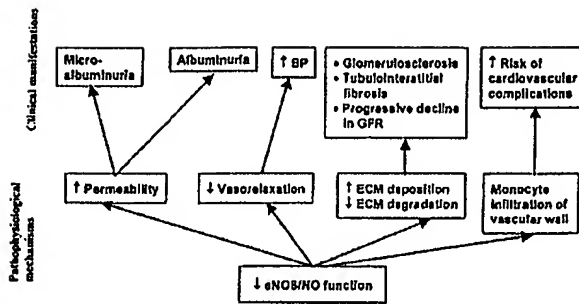
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**Figure 1.** Clinical manifestations of diabetic nephropathy and potential role of ECD, defined as dysregulation of eNOS/NO availability, in pathophysiology of these clinical presentations. BP indicates blood pressure; GFR, glomerular filtration rate.

antithrombotic properties; perturbed angiogenic capacity; inappropriate regulation of vascular smooth muscle tonicity, proliferative capacity, and migratory properties; perturbed synthetic functions; and deterrent of neutrophils and monocytes from diapedesis. The pathophysiology of endothelial cells characterized by these abnormalities, expressed at various degrees, is emerging as a hallmark of several highly prevalent cardiovascular and renal diseases, including diabetes mellitus, as well as their complications.

Although several markers of ECD have been proposed (elevated circulating levels of von Willebrand factor, plasminogen activator inhibitor [PAI]-1, some adhesion molecules, isoprostane, and thrombomodulin [reviewed in Reference 11]),<sup>11</sup> endothelium-dependent vasorelaxation has remained the gold standard in assessing endothelial function and dysfunction.<sup>12</sup> The demonstration of a paradoxical vasoconstriction in atherosclerotic coronary arteries in response to infusion of acetylcholine, a clinical equivalent of Furchgott's and Zawadzki's<sup>13</sup> observation of endothelium-dependent vasorelaxation and its reversal in denuded vessels, pointed to the pivotal role of endothelial nitric oxide synthase (eNOS) in the pathogenesis of ECD. Indeed, accumulated evidence suggests that many of the above-mentioned aspects of ECD are intimately linked to the expression and function of this enzyme. In particular, nitric oxide (NO) generation inhibits platelet aggregation; similarly, adhesion of leukocytes to the vascular endothelium is inhibited by NO.<sup>14–20</sup> Endothelial regulation of vascular smooth muscle relaxation, proliferation, and migration is in part governed by the integrity of the L-arginine–eNOS–NO system.<sup>21–23</sup> In addition, vascular/endothelial permeability and some synthetic functions of endothelial cells have been linked to the activity of eNOS (reviewed in Reference 11). Hence, NO production or availability can regulate diverse functions in endothelial cells per

se and their interaction with circulating formed elements (both inflammatory and thrombotic interactions) and vascular smooth muscle cells. In fact, recent findings from Casellas's laboratory (Bouriquet et al<sup>24</sup>) demonstrated that in the absence of hyperlipidemia, inhibition of eNOS alone is sufficient to induce the deposition of Sudan black-positive lipid droplets in arcuate and interlobular renal but not in afferent arterioles, resulting in increased vascular wall thickness. This is an important demonstration of the role of NO generation in atherosclerotic damage to the medium-size renal arteries.

The main thesis of this review, therefore, is that the pathophysiological basis for the Steno hypothesis is endothelial cell dysfunction and, specifically, the dysfunction of the eNOS/NO system. Below, we shall consider two phases of its development: the initiation phase and the maintenance phase.

### Initiation Phase of Endothelial Cell Dysfunction

What triggers ECD in diabetes mellitus? Numerous epidemiological and pathophysiological studies point to the importance of hyperglycemia in development of macrovascular and microvascular complications.<sup>25</sup>

One of the early alterations observed in diabetes mellitus that may lead to initiation of endothelial cell dysfunction is the decreased bioavailability of NO. In a series of studies performed in collaboration with S. Gross (Weill Medical College), we have demonstrated that supraphysiological concentrations of D-glucose (30 mmol/L) are capable of scavenging NO.<sup>26</sup> Specifically, we demonstrated that acute exposure of human endothelial cells to glucose, at levels found in plasma of diabetic patients, results in a significant blunting of NO responses to the eNOS agonists bradykinin and A23187. Monitoring of NO generation by purified recombinant bovine eNOS *in vitro*, with the use of amperometric electrochemical detection and an NO-selective porphyrinic microelectrode, showed that glucose causes a progressive and concentration-dependent attenuation of detectable NO. Addition of glucose to pure NO solutions similarly elicited a sharp decrease in NO concentration, indicating that glucose promotes NO loss. Electrospray ionization tandem mass spectrometry, using negative ion monitoring, directly demonstrated the occurrence of a covalent reaction involving unitary addition of NO (or a derived species) to glucose. This effect of glucose may account for the acute hypertensive response to hyperglycemia,<sup>27,28</sup> as well as for multiple cellular effects, as detailed in Table 1. In addition, extrapolating from these *in vitro* data, each episode of hyperglycemia, as transient as it might be, will lead to the temporary decrease in the bioavailability of

**TABLE 1.** Cellular Consequences of NO Scavenging by Glucose

Endothelium	Transient dysfunction (loss of tonic vasorelaxing function, antithrombotic properties, impaired deterrent of polymorphonuclear neutrophils and monocytes from adhesion and diapedesis, suppressed responsiveness to angiogenic cues, and defective wound healing)
Pancreatic $\beta$ -cells	Defective glucose-stimulated insulin secretion (early phases are NO-dependent)
Macrophages	Defective response to lipopolysaccharide stimulation
Neurons	Reduced cGMP generation (preconditioning for diabetic neuropathy?)
Skeletal muscle	Impaired insulin-stimulated glucose utilization

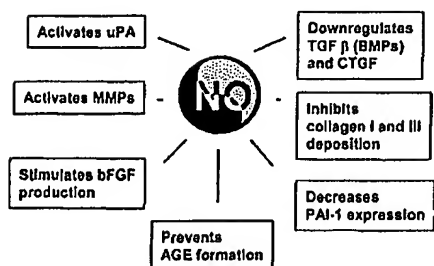


Figure 2. Relevant targets of NO and the consequences of NO deficiency. uPA indicates urokinase type plasminogen activator; MMPs, matrix metalloproteinases; bFGF, basic fibroblast growth factor; TGF, transforming growth factor; BMPs, bone morphogenic proteins; and CTGF, connective tissue growth factor.

NO and the reversible impairment of NO-dependent functions of the endothelium, as illustrated in Figure 2. It should be emphasized that glucose-NO adducts formed are unstable and gradually release bioactive NO, but this messenger molecule will be then delivered to inappropriate targets at the wrong time. The proposed consequences of hyperglycemic episodes, at the level of the vascular endothelium, are summarized in Figure 3, which illustrates the transient decline in NO bioavailability and transient proatherogenic changes in the vascular wall. Another major consequence of the elevated plasma glucose levels, which, however, shows poor reversibility and in fact has a cumulative dependence on hyperglycemia, is the formation of advanced glycation end-products (AGEs) (Figure 4).

### Transition From Initiation to Maintenance Phase of Endothelial Dysfunction

Several arguments exist that link the formation of AGEs to the maintenance phase of endothelial cell dysfunction. Previous studies by Bucala et al<sup>29</sup> showed that AGEs have an NO-scavenging effect. AGEs are considered among the leading causes of diabetic complications, especially in the development of atherosclerotic vascular disease (see References 30 to 34 and references therein).<sup>30-34</sup> Their mode of action is linked to changes in physicochemical properties of matrix proteins such as collagen-to-collagen cross-linking and tissue rigidity, leading to decreased solubility and susceptibility of proteins to enzymatic digestion. In fact, infusion of AGEs to nondiabetic animals can reproduce many vascular

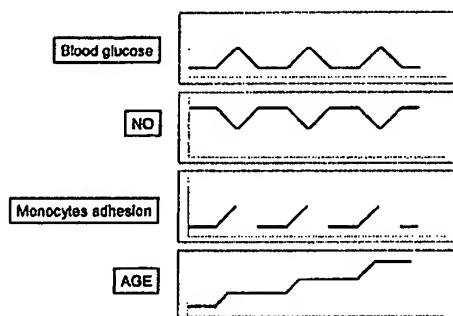


Figure 3. Schematic view of hyperglycemia-induced transient and cumulative effects on vascular wall.

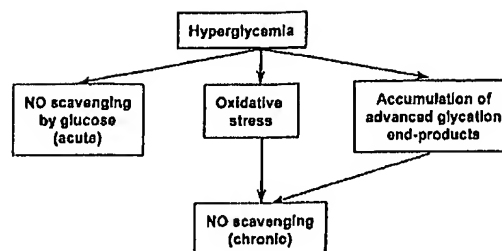


Figure 4. Summary of proposed pathophysiological mechanisms acting during initiation phase of ECD.

complications of diabetes. Recent data have demonstrated that AGEs consume endothelium-derived NO, thus compromising vasodilatory responses and diminishing antiproliferative action of NO.<sup>29</sup> Indeed, substantial literature exists on the stimulation of NOS in diabetic animals and humans, whereas the biological effects of NO are deficient (reviewed in References 35 to 38).<sup>35-38</sup> These findings of NO quenching by AGEs may explain the observed deficiency of angiogenic responses, which require basal NO production, at sites of interstitial fibrosis. In addition, this functional NO deficiency may complement other factors in stimulating the proliferation of fibroblasts and the accumulation of the extracellular matrix, both of which are important contributors to the progression of diabetic nephropathy (reviewed in Reference 39).<sup>39</sup> Collectively, these findings provide the conceptual basis to link AGEs with the development of the maintenance phase of endothelial cell dysfunction. We shall present our data, further confirming and developing these observations.

### Maintenance Phase of Endothelial Cell Dysfunction

Endothelial cells cultured in 3D glycosylated collagen I gels showed delayed capillary cord branching, examined with a previously described *in vitro* angiogenesis assay.<sup>40</sup> This branching incompetence was chronologically associated with the induction of several genes, as detected by a differential display approach (Table 2). One of such genes was PAI-1, a well-established marker of endothelial dysfunction in several pathological conditions, including diabetes mellitus. Studies that used exogenous PAI-1 or neutralizing antibodies to PAI-1 have demonstrated that it is critically involved in the observed delay in capillary cord branching *in vitro*.<sup>41</sup>

To confirm the role of PAI-1 *in vivo*, angiogenesis assays were performed in PAI-1<sup>-/-</sup> mice. Aortic explants obtained from these mice<sup>42,43</sup> showed uninhibited vascular sprouting in 3D cultures in glycosylated collagen or matrigel, as opposed to the vessels obtained from wild-type mice. Furthermore, aortic explants obtained from streptozotocin-induced diabetic (STZ) mice or rats, although indistinguishable from control animals 4 weeks after STZ injection, showed defective capillary sprouting by 8 weeks. Interestingly, when STZ diabetes was modeled in PAI-1<sup>-/-</sup> mice, explanted aortic cultures showed a much improved angiogenic capacity compared with wild-type animals (S. Brodsky, unpublished observations). Collectively, these data demonstrate that PAI-1 is an early-response gene induced in endothelial cells presented with

**TABLE 2. Polymerase Chain Reaction–Selected Subtraction Search for Differentially Displayed cDNA Clones in Human Umbilical Vein Endothelial Cells Exposed to Glycated Collagen I**

Genes	No. of Clones	Partial Sequence of Insert
PAI-1	3	TGCCCTACCACGGGACACCTCA
Human elongation factor 1- $\alpha$	3	AGCTCCAGCAGCCTTCTGTCCACT
Mitochondrion cytochrome oxidase, subunit II	2	GTACCATGGTGGCCAATTGATTG
Thymosin $\beta$ -4	2	ACTCGTGCCTCGCTTCGCTTTTC
Lipocortin-2 pseudogene	2	TGTGGTGGAGATGACTGAAGCCCG
Syntenin	1	TACTTGGTCCCAATCTCAGACCA
Heat shock protein 90	1	CAGCGCAGGGGCTTCTGCTGAGGG
Claudin 11	1	CAAGTAAATGACCTTGAAGGAAG
Unknown yet	1	GAGCAGCTCTACTTAATGCAATTCT
Others	4	

glycated collagen and that the product of this gene is causally involved in the impaired angiogenic competence. Together with the existing clinical studies incriminating PAI-1 in diabetic complications,<sup>11,44,45</sup> it is conceivable that PAI-1 is an important contributor to the maintenance of endothelial cell dysfunction. The question is: Does PAI-1 affect the eNOS/NO system?

Recent studies (S. Brodsky, unpublished observations) demonstrated that treatment of cultured human umbilical vein endothelial cells with the constitutively active PAI-1 resulted in the reversible decrease of immunodetectable eNOS. This was associated with the reduced ability of endothelial cells to generate NO in response to bradykinin or A23187. The similar phenomenon was observed in endothelial cells cultured on the surface of glycated collagen or matrigel. This series of observations strongly suggests that the maintenance phase of endothelial cell dysfunction in diabetes mellitus could be attributed not only to the scavenging of NO by elevated glucose or AGEs but to the chronic suppression of its expression and function by the activated PAI-1.

Assuming the latter takes place in vivo, the state of chronic endothelial NO deficiency will have a broad range of functional alterations, as schematically shown in Figure 5. Specifically, eNOS/NO deficiency should lead to the impaired balance between the matrix deposition and degradation, result in activation of transforming growth factor- $\beta$  and connective tissue growth factor, promote proatherosclerotic changes in the vascular wall, accelerate formation of AGEs, impair

angiogenic remodeling of the vascular bed to the ischemic tissues, and interfere with insulin secretion and glucose utilization by skeletal muscles (both processes NO-dependent). All these sequels of eNOS/NO deficiency have clear-cut relevance to the progression of diabetic nephropathy.

### Conclusions

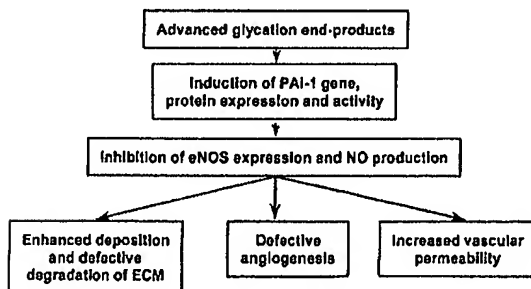
The hypothesis presented herein ascribes clinical manifestations and their respective pathophysiological mechanisms to the development of endothelial cell dysfunction. The trigger for its development is hyperglycemia per se, but the maintenance phase is tightly linked to the accumulation of AGEs. The pivotal function of endothelial cells perturbed during the initiation and maintenance phases is eNOS/NO production or availability. We propose that the initiation event(s) is linked to glucose scavenging of NO during transient episodes of hyperglycemia. At the maintenance phase, however, eNOS expression and function may be perturbed chronically, thus leading to the persistent dysfunction of the vascular endothelium. This hypothesis, while stemming from the Steno hypothesis on endothelial pathology preceding diabetic complications, puts forward eNOS/NO dysfunction as the critical variable responsible for the initiation and maintenance of endothelial dysfunction. Shifting the emphasis from *N*-deacetylase to the eNOS/NO system may have important implications for therapy of diabetic complications, including diabetic nephropathy.

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**Figure 5. Summary of proposed pathophysiological mechanisms acting during maintenance phase of ECD.**

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